

FR167653 inhibits fibronectin expression and apoptosis in diabetic glomeruli and in high-glucose-stimulated mesangial cells

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Submitted 31 December 2007; accepted in final form 2 June 2008

Jung D-S, Li JJ, Kwak S-J, Lee SH, Park J, Song YS, Yoo T-H, Han SH, Lee JE, Kim DK, Moon SJ, Kim YS, Han DS, Kang S-W. FR167653 inhibits fibronectin expression and apoptosis in diabetic glomeruli and in high-glucose-stimulated mesangial cells. *Am J Physiol Renal Physiol* 295: F595–F604, 2008. First published June 4, 2008; doi:10.1152/ajprenal.00624.2007.—Previous in vitro studies suggest that the p38 MAPK pathway may be involved in the pathogenesis of diabetic nephropathy, but the consequences of the inhibition of the p38 MAPK pathway have not been well elucidated in diabetic (DM) glomeruli. This study was undertaken to investigate the effect of p38 MAPK inhibitor, FR167653, on fibronectin expression and apoptosis in DM glomeruli and in high-glucose-stimulated mesangial cells (MC). In vivo, 32 Sprague-Dawley rats were injected with diluent (control, $N = 16$) or streptozotocin intraperitoneally (DM, $N = 16$). Eight rats from each group were treated with FR167653 for 3 mo. In vitro, rat MC were exposed to medium containing 5.6 mM glucose or 30 mM glucose [high glucose (HG)] with or without 10^{-6} M FR167653 for 24 h. Fibronectin mRNA and protein expression were determined by real-time PCR and Western blot, respectively. Western blot for apoptosis-related molecules, terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling assay, and Hoechst 33342 staining were performed to determine apoptosis. FR167653 ameliorated the increases in fibronectin-to-GAPDH mRNA ratio and protein expression in DM glomeruli by 89 and 79% and in HG-stimulated MC by 70 and 91%, respectively ($P < 0.05$). Under diabetic conditions, Bcl-2 protein expression was decreased, whereas cleaved caspase-3 protein expression was increased ($P < 0.05$), and these changes were inhibited by FR167653 treatment. Apoptotic cells were also significantly increased in DM glomeruli and in HG-stimulated MC ($P < 0.05$), and FR167653 ameliorated these increases in apoptotic cells, both in vivo and in vitro. In conclusion, these findings suggest that the inhibition of the p38 MAPK pathway has a beneficial effect on the development of diabetic nephropathy by inhibiting the increase in fibronectin expression and apoptosis.

p38 mitogen-activated protein kinase; fibrosis; apoptosis; diabetic nephropathy

THE MOLECULAR AND CELLULAR mechanisms responsible for diabetic nephropathy remain incompletely resolved. While studies indicate involvement of hyperglycemia via the stimulation of growth factor-induced cellular hypertrophy (48), increased

production of extracellular matrix (ECM) protein (2), and decreased production of matrix-degrading proteinases (20), the underlying signal transduction mechanisms mediating these processes have been less well explored.

Numerous studies reveal protein kinase C (PKC) activation in diabetic glomeruli (5) and in mesangial cells cultured under high-glucose conditions (42). PKC propagates the physiological responses of receptor-ligand interactions via an array of downstream signals, such as mitogen-activated protein kinases (MAPKs). p38 MAPK, a member of the MAPK family, is known to be activated in response to stress signals, such as proinflammatory cytokines (28, 34), ultraviolet irradiation (34), osmolality changes (28, 34), and oxidative stress (4, 28), leading to apoptosis (43), prostanoid production (22), and other cellular dysfunctions (8). Since hyperosmolality and oxidant stress characterize the diabetic state, p38 MAPK has been posited to mediate the pathogenesis of diabetic complications. Increased p38 MAPK activity has been observed in the aorta and the glomeruli of diabetic rats and in vascular smooth muscle cells and mesangial cells cultured under high-glucose conditions, along with increased ECM synthesis (9, 13, 14, 30). Oxidant stress, a strong activator of the p38 MAPK pathway, was a proximate event in high-glucose-induced mesangial cell apoptosis (12). In addition, methylglyoxal, a potent precursor of advanced glycation end products, induced apoptosis in cultured mesangial cells via the p38 MAPK pathway (25). These in vitro findings suggest that the p38 MAPK pathway may be involved in the pathogenesis of ECM accumulation and mesangial cell apoptosis under diabetic conditions. However, the direct relationship between apoptosis and the p38 MAPK pathway has never been documented in diabetic glomeruli and in mesangial cells exposed to high glucose. In addition, the consequences of the inhibition of the p38 MAPK pathway have not been well elucidated in diabetic glomeruli.

In this study, we examined the effect of FR167653 on the p38 MAPK pathway in isolated glomeruli from diabetic rats and in mesangial cells cultured under high-glucose conditions. In addition to p38 MAPK, we also studied the effect of FR167653 on the following: 1) cAMP-responsive element (CRE) binding protein (CREB), a transcription factor that is known to be under control of p38 MAPK; 2) fibronectin, a key

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ECM protein produced by mesangial cells; and 3) apoptosis-related molecules, such as Bax, Bcl-2, and caspase-3.

METHODS

Animals. All animal studies were conducted under an approved protocol. Thirty-two male Sprague-Dawley rats, weighing 250–280 g (8–9 wk old), were injected either with diluent [$n = 16$, control (C)] or with 65 mg/kg streptozotocin intraperitoneally [$n = 16$, diabetes (DM)]. Eight rats from each group were treated with 2 mg·kg⁻¹·day⁻¹ of FR167653 (kindly donated from Astellas Pharma, Tokyo, Japan) (diluted in 0.9% saline) by intramuscular injection (C+FR, DM+FR) for 3 mo, while C and DM rats were injected with similar volume of 0.9% saline. Since the previous study has shown that glomerular p38 MAPK activity was increased for the first 3 mo after DM induction by streptozotocin (15), we decided to administer FR167653 for 3 mo in this study. In addition, the dose of FR167653 was determined based on the previous study by Lu et al. (26). Rats were housed in a temperature-controlled room and were given free access to water and standard laboratory chow during the 3-mo study period.

Body weights were checked monthly, and kidney weights were measured at the time of death. Systolic blood pressure was measured by tail-cuff plethysmography at 3 mo. Serum glucose and 24-h urinary albumin were also measured at the time of death. Blood glucose was measured by glucometer, and 24-h urinary albumin excretion was determined by ELISA (Nephra II, Exocell, Philadelphia, PA).

Glomerular isolation. Glomeruli were isolated by sieving. Purity of the glomerular preparation was greater than 98%, as determined by light microscopy.

Mesangial cell culture. Isolated glomeruli from male Sprague-Dawley rats were incubated in collagenase and trypsin-EDTA (Gibco Laboratories, Bethesda, MD), as described previously (1). Mesangial cells were maintained in RPMI 1640 media (Sigma Chemical, St. Louis, MO), supplemented with L-glutamine, 7 mM HEPES, and 10% fetal bovine serum (Gibco Laboratories), and incubated at 37°C in humidified 5% CO₂ in air.

To determine the effect of FR167653 on high-glucose-induced fibronectin and apoptosis-related molecule expression, rat mesangial cells were serum restricted for 24 h, and the medium was changed to serum-free RPMI medium containing normal glucose (5.6 mM; NG), high glucose (30 mM; HG), or NG + mannitol (24.4 mM) with or without 10⁻⁶ M FR167653. The concentrations of FR167653 used in this study were determined based on preliminary experiments. Cells were harvested for either RNA or protein at 24 h after the media change.

Total RNA extraction and reverse transcription. Total RNA was extracted from isolated glomeruli as previously described (15), and first-strand cDNA was made by using a Boehringer Mannheim cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany). Two micrograms of total RNA extracted from sieved glomeruli were reverse transcribed using 10 µM random hexanucleotide primer, 1 mM dNTP, 8 mM MgCl₂, 30 mM KCl, 50 mM Tris·HCl, pH 8.5, 0.2 mM dithiothreitol, 25 units RNase inhibitor, and 40 units avian myeloblastosis virus reverse transcriptase. The mixture was incubated at 30°C for 10 min and 42°C for 1 h, followed by inactivation of the enzyme at 99°C for 5 min. Cellular RNA from each plate was similarly reverse transcribed.

Real-time polymerase chain reaction. The primers used for fibronectin and GAPDH amplification were as follows: fibronectin sense, 5'-TGACAAGTCCGCTAGACCTGG-3', antisense 5'-TACT-GGTGTAGGTGTGGCCG-3'; and GAPDH sense 5'-CGAGAAT-GGGAAGCTTGTCATC-3', antisense 5'-CGGCCTACCCCATTTG-3'. cDNAs synthesized from 20 ng RNA of glomeruli or cells were used per reaction for amplification.

Using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA), polymerase chain reaction (PCR) was performed with a total volume of 20 µl in each well, containing 10 µl

of SYBR Green PCR Master Mix (Applied Biosystems), 5 µl of cDNA, and 5 pmol of sense and antisense primers. Primer concentrations were determined by preliminary experiments that analyzed the optimal concentrations of each primer. Each sample was run in triplicate in separate tubes to permit quantification of the fibronectin gene expression normalized to the GAPDH. The PCR conditions used were as follows: for GAPDH, 35 cycles of denaturation at 94.5°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min; and for fibronectin, 38 cycles of denaturation at 94.5°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s. An initial heating at 95°C for 9 min and a final extension at 72°C for 7 min were performed for all PCR reactions. After real-time PCR, the temperature was increased from 60 to 95°C at a rate of 2°C/min to construct a melting curve. A control without cDNA was run in parallel with each assay. The cDNA content of each specimen was determined using a comparative threshold cycle (C_T) method with 2^{-ΔΔC_T}. The results were given as relative expression normalized to the GAPDH gene and expressed in arbitrary units. Signals from C glomeruli and NG cells were assigned a relative value of 1.0. In pilot experiments, PCR products run on agarose gels revealed a single band.

Western blot analysis. Sieved glomeruli and cells harvested from plates were lysed in sodium dodecyl sulfate (SDS) sample buffer [2% SDS, 10 mM Tris·HCl, pH 6.8, 10% (vol/vol) glycerol], treated with Laemmli sample buffer, heated at 100°C for 5 min, and electrophoresed in an 8% acrylamide denaturing SDS-polyacrylamide gel. Proteins were then transferred to a Hybond-ECL membrane using a Hoeffer semidry blotting apparatus (Hoeffer Instruments, San Francisco, CA), and the membrane was then incubated in blocking buffer A (1 × PBS, 0.1% Tween 20, and 8% nonfat milk) for 1 h at room temperature, followed by an overnight incubation at 4°C in a 1:1,000 dilution of polyclonal antibodies to p38 MAPK, phospho-specific p38 MAPK, CREB, phospho-specific CREB, extracellular signal-regulated kinase-1/2 (ERK1/2), phospho-specific ERK1/2, c-Jun NH₂-terminal kinase (JNK), phospho-specific JNK, PKC (New England Biolaboratories, Beverly, MA), extracellular domain of fibronectin, Bax, Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), active fragments of caspase-3, total caspase-3 (Cell Signaling, Beverly, MA), or β-actin (Santa Cruz Biotechnology). The membrane was then washed once for 15 min and twice for 5 min in 1 × PBS with 0.1% Tween 20. Next, the membrane was incubated in buffer A containing a 1:1,000 dilution of horseradish peroxidase-linked goat anti-rabbit IgG (Amersham Life Science, Arlington Heights, IL). The washes were repeated, and the membrane was developed with a chemiluminescent agent (ECL; Amersham Life Science), and the band densities were measured using TINA image software (Raytest, Straubenhardt, Germany).

Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling assay and Hoechst 33342 staining. In addition to the changes in the expression of apoptosis-related molecules, apoptosis was also identified within glomeruli by terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) using commercially available kit (Chemicon International, Temecula, CA) and in cultured mesangial cells seeded on coverslips by Hoechst 33342 (Molecular Probes, Eugene, OR) staining. Apoptosis was defined as TUNEL-positive cells within glomeruli and the presence of nuclear condensation on Hoechst staining. TUNEL-positive glomerular cells in formalin-fixed renal tissue and the percentage of mesangial cells with nuclear condensation were determined by examining at least 30 glomeruli and 300 cells per condition, respectively, at ×400 magnification.

Immunohistochemistry. Slices of kidney for immunohistochemical staining were snap-frozen in optimal cutting temperature solution, and 4-µm sections of tissues were utilized. Slides were fixed in acetone for 10 min at 4°C, air dried for 10 min at room temperature, and blocked with 10% donkey serum for 20 min at room temperature. For fibronectin staining, the primary polyclonal antibody to the extracellular domain of fibronectin (Santa Cruz Biotechnology) was diluted in

1:100 with 2% casein in BSA and was applied for overnight incubation at room temperature. After washing, a secondary goat anti-rabbit antibody was added for 20 min, and the slides were then washed and incubated with a tertiary rabbit-peroxidase-antiperoxidase complex for 20 min. 3,3'-Diaminobenzidine was added for 2 min, and the slides were counterstained with hematoxylin. A semiquantitative score for measuring the intensity of fibronectin staining within glomeruli was determined by examining 30 glomeruli in each section and by digital image analysis (MetaMorph version 4.6r5, Universal Imaging, Downingtown, PA), as previously described (16).

Statistical analysis. All values are expressed as means \pm SE. Statistical analysis was performed using the statistical package SPSS for Windows version 11.0 (SPSS, Chicago, IL). Results were analyzed using the Kruskal-Wallis nonparametric test for multiple comparisons. Significant differences by the Kruskal-Wallis test were further confirmed by the Mann-Whitney *U*-test. *P* values <0.05 were considered to be statistically significant.

RESULTS

Animal data. All animals gained weight over the 3-mo experimental period, but weight gain was highest in C rats ($P < 0.01$). The ratio of kidney weight to body weight in DM rats ($1.46 \pm 0.09\%$) was significantly higher than that in C ($0.57 \pm 0.05\%$), C+FR (0.53 ± 0.06) ($P < 0.01$), and DM+FR rats ($0.93 \pm 0.07\%$) ($P < 0.05$). The mean blood glucose levels of C, C+FR, DM, and DM+FR rats were 109.9 ± 6.5 , 112.5 ± 8.3 , 487.5 ± 14.4 , and 479.0 ± 13.2 mg/dl, respectively ($P < 0.01$). Compared with the C group (0.35 ± 0.06 mg/day), 24-h urinary albumin excretion was significantly higher in the DM group (1.87 ± 0.26 mg/day, $P < 0.05$), and FR167653 treatment significantly reduced albuminuria in DM rats (0.77 ± 0.16 mg/day, $P < 0.05$).

p38 MAPK activity and protein expression. Figure 1 shows a representative Western blot of equal amounts of protein from the lysates of sieved glomeruli from the four groups. The blot was first probed with an antibody to phospho-specific p38

MAPK and then stripped and probed with an antibody that recognizes total p38 MAPK. Phospho-specific (activated) p38 MAPK levels were significantly greater in the DM compared with C and C+FR glomeruli ($P < 0.05$). FR167653 treatment nearly normalized the increase in glomerular p38 MAPK activity in DM rats ($P < 0.05$). In contrast, no significant differences were found in total p38 MAPK expression among the four groups.

The effects of FR167653 on p38 MAPK activity and protein expression in HG-stimulated mesangial cells are shown in Fig. 2. HG activated the p38 MAPK pathway in cultured mesangial cells, and this HG-induced p38 MAPK activity was ameliorated by 79% with 10^{-6} M FR167653 treatment ($P < 0.01$). In contrast, FR167653 had no effect on total p38 MAPK protein expression in cultured mesangial cells.

On the other hand, we tested whether the inhibitory effect of FR167653 was specific on the p38 MAPK pathway. As seen in Fig. 3, the increases in ERK1/2 and JNK activities and PKC protein expression in HG-stimulated mesangial cells were not affected by FR167653 treatment.

CREB activity and protein expression. To determine whether activation of the p38 MAPK pathway could induce parallel increases in the activity of a p38 MAPK target transcription factor, we examined the activity and protein expression of CREB. The representative blot in Fig. 1 shows that phospho-specific (activated) CREB expression was significantly higher in DM relative to C and C+FR glomeruli ($P < 0.01$). Similar to p38 MAPK activity, FR167653 treatment ameliorated the increase in glomerular CREB activity in DM rats ($P < 0.05$). In contrast, there were no significant differences in total CREB levels among the four groups.

The effects of FR167653 on CREB activity and protein expression in HG-stimulated mesangial cells were similar to those on p38 MAPK activity and protein expression. HG

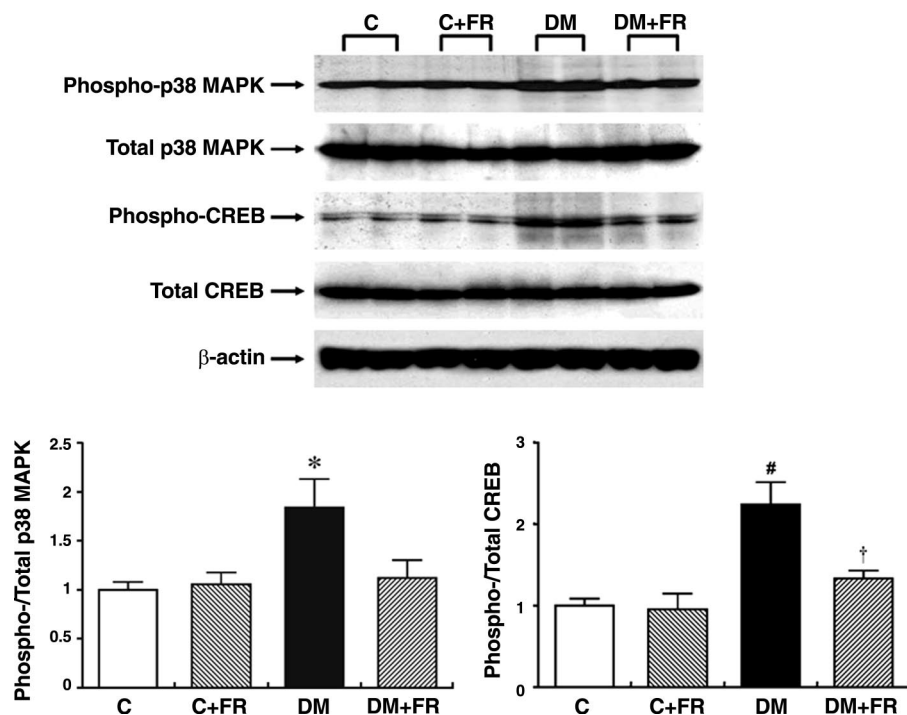
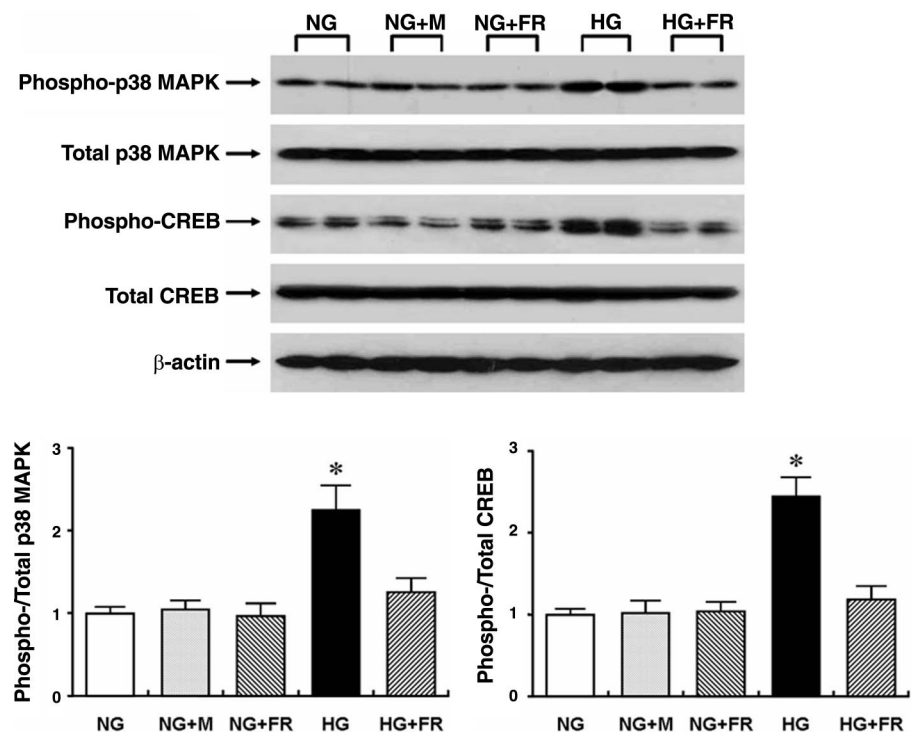


Fig. 1. Representative Western blot of glomerular phospho-specific and total p38 mitogen-activated protein kinase (MAPK) and cAMP-responsive element binding protein (CREB) in control (C), C + FR167653-treated (FR), diabetes (DM), and DM+FR groups (representative of four blots). Phospho-specific p38 MAPK-to-total p38 MAPK and phospho-specific CREB-to-total CREB ratios were significantly greater in DM compared with C (1.8-fold and 2.2-fold, respectively) and C+FR glomeruli, and FR167653 treatment nearly normalized the increases in glomerular p38 MAPK and CREB activities in the DM group. In contrast, there were no differences in total p38 MAPK, total CREB, and β -actin protein expression among the four groups. * $P < 0.05$ vs. other groups; # $P < 0.01$ vs. C and C+FR groups; † $P < 0.05$ vs. DM group.

Fig. 2. Representative Western blot of phospho-specific and total p38 MAPK and CREB in cultured mesangial cells (representative of four blots). High glucose (HG) significantly induced phospho-specific p38 MAPK and CREB levels in cultured mesangial cells, and these increments in p38 MAPK and CREB activities were ameliorated by FR167653 treatment. In contrast, FR167653 and mannitol (M) had no effects on total p38 MAPK, total CREB, and β -actin protein expression in cultured mesangial cells. NG, normal glucose. * $P < 0.01$ vs. other groups.



induced CREB activation in mesangial cells, and this HG-induced CREB activity was ameliorated by 10^{-6} M FR167653 treatment ($P < 0.01$). In contrast, FR167653 had no effect on total CREB levels in cultured mesangial cells (Fig. 2).

Fibronectin mRNA and protein expression. To correlate our observations to evolving diabetic nephropathy, we examined the mRNA and protein expression of fibronectin, a key ECM protein of mesangial cells. The glomerular fibronectin mRNA expression was significantly increased in DM compared with C and C+FR rats ($P < 0.05$). On the other hand, there was no difference in GAPDH mRNA expression among the four groups (data not shown). The fibronectin-to-GAPDH mRNA ratio and fibronectin protein expression were 2.1- and 1.9-fold higher, respectively, in DM relative to C glomeruli ($P < 0.05$), and these increments were inhibited by 89 and 79%, respectively, with FR167653 treatment ($P < 0.05$) (Fig. 4).

HG also induced fibronectin mRNA and protein expression in cultured mesangial cells. The cellular fibronectin mRNA expression was significantly increased in HG-stimulated mesangial cells compared with NG cells ($P < 0.01$). On the other hand, there was no difference in GAPDH mRNA expression among the groups (data not shown). The fibronectin-to-GAPDH mRNA ratio and fibronectin protein expression were 2.3- ($P < 0.01$) and 1.7-fold higher ($P < 0.05$), respectively, in mesangial cells exposed to HG, and FR167653 treatment ameliorated these increments in fibronectin expression in HG cells (Fig. 5).

Bax, Bcl-2, and active fragments of caspase-3 protein expression. Bcl-2 protein expression was significantly decreased, while active fragments of caspase-3 protein expression were significantly increased in DM glomeruli and HG-stimulated mesangial cells ($P < 0.01$), and these

changes were inhibited by FR167653, both in vivo and in vitro. On the other hand, Bax protein expression was significantly increased in DM compared with C glomeruli, and FR167653 treatment abrogated this increment in DM glomeruli. An increase in Bax protein expression was also observed in HG-stimulated mesangial cells but did not reach statistical significance (Figs. 6 and 7).

TUNEL assay and Hoechst 33342 staining. Apoptotic cells assessed by TUNEL assay and Hoechst 33342 staining were significantly increased in DM glomeruli ($P < 0.01$) and HG-stimulated mesangial cells ($P < 0.05$) compared with C glomeruli and NG cells, respectively, and these increases in apoptotic cells were ameliorated by FR167653, both in vivo and in vitro ($P < 0.05$) (Fig. 8).

Immunohistochemistry. Immunohistochemical staining for glomerular fibronectin confirmed the Western blot findings. Glomerular fibronectin staining within mesangial regions was significantly stronger in DM compared with C rats, and FR167653 treatment inhibited the increase in glomerular fibronectin accumulation in DM rats (Fig. 9). The mean semiquantitative staining scores for glomerular fibronectin were significantly higher in DM (86.3 ± 11.1) compared with C rats (28.5 ± 5.8) ($P < 0.05$), and FR167653 treatment attenuated this increment in DM glomeruli (41.1 ± 6.9) ($P < 0.05$).

DISCUSSION

Diabetic milieu is known to activate the p38 MAPK pathway in various cells and organs (9, 13, 14, 16, 30, 44); however, the consequences of inhibiting p38 MAPK activation under diabetic conditions have not been well explored in diabetic nephropathy. In this study, we demonstrate for the first time that fibronectin expression and apoptosis in diabetic glomeruli and

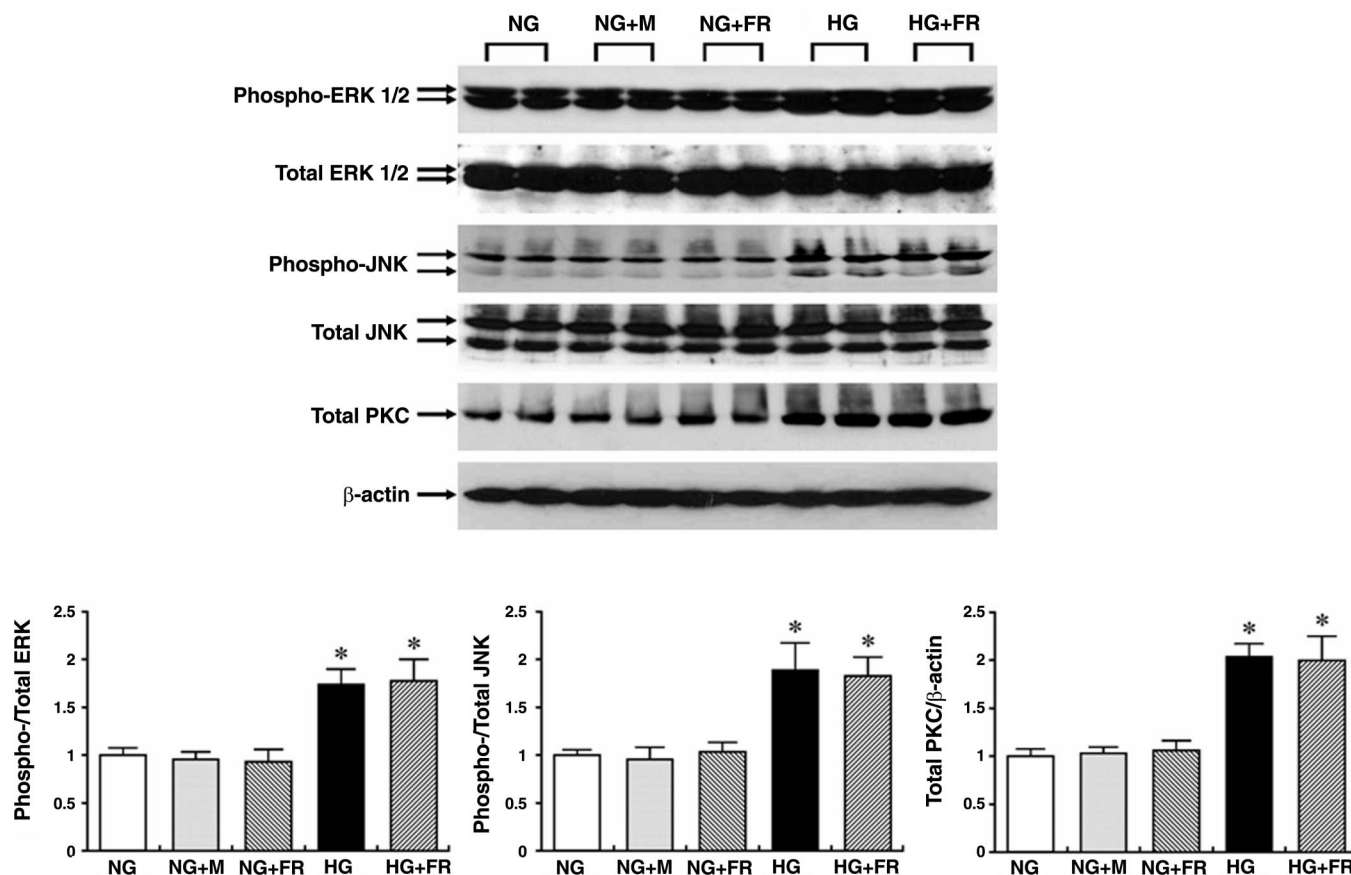


Fig. 3. Representative Western blot of phospho-specific and total extracellular signal-regulated kinase (ERK)-1/2, c-Jun NH₂-amino terminal kinase (JNK), and total protein kinase C (PKC) in cultured mesangial cells (representative of four blots). FR167653 treatment did not affect the increases in ERK1/2 and JNK activities and total PKC protein expression in HG-stimulated mesangial cells. * $P < 0.05$ vs. NG, NG+M, and NG+FR groups.

HG-stimulated mesangial cells were ameliorated with the administration of FR167653, a p38 MAPK inhibitor.

p38 MAPK is a member of the MAPK family and is known as a "stress-activated kinase," along with JNK (7, 24, 36). Biochemical studies have demonstrated that the p38 MAPK signaling pathway activates various transcription factors, including CREB (10). CREB is a member of a transcription factor family that converts rapid and transient signals into long-term changes in gene expression by binding to the CRE. Since the fibronectin promoter contains a CRE (3), activated CREB can bind the CRE portion of the fibronectin gene, leading to fibronectin mRNA expression. Kreisberg et al. (23) have demonstrated that activation of PKC by HG plus transforming growth factor- β or phorbol myristate acetate induced phosphorylation of CREB, resulting in the stimulation of fibronectin transcription in mesangial cells in vitro. These studies suggest that activation of p38 MAPK in diabetic glomeruli and in HG-stimulated mesangial cells is involved in fibronectin accumulation. We now report that treatment with a p38 MAPK inhibitor in vivo and in vitro ameliorated p38 MAPK phosphorylation and that the increase in fibronectin expression was subsequently inhibited.

FR167653 was originally developed as a dual inhibitor of interleukin-1 and tumor necrosis factor (TNF)- α production (47). Previous studies have demonstrated that FR167653 ameliorated ischemia-reperfusion injury in the lung, pancreas, and

kidney, possibly by inhibition of proinflammatory cytokine production (18, 31, 46). In rats with nephrotoxic serum nephritis, an animal model of human crescentic glomerulonephritis, urinary excretion and renal expression of monocyte chemoattractant protein-1 were decreased by FR167653 (41). FR167653 also reduced the renal expression of monocyte chemoattractant protein-1 and TNF- α in an animal model of human lupus erythematosus, resulting in prolonged survival and attenuated renal pathological changes (11). Since glomerular infiltration of leukocytes has a major role in the pathogenesis of nephrotoxic serum nephritis and lupus nephritis, the effects of FR167653 seem to be mediated by its anti-inflammatory actions, i.e., inhibition of cytokine production by monocytes and macrophages. Very recently, Moriwaki et al. (29) have demonstrated that urinary albumin excretion is decreased in FR167653-treated diabetic rats, along with modest decrease in the urinary excretion of TNF- α , suggesting an important role of TNF- α in the pathogenesis of diabetic nephropathy. In that study, however, the pathological changes, such as ECM accumulation or inflammatory cell infiltration, were not investigated. Since cellular infiltration within glomeruli is not extensive in 3-mo diabetic rat compared with tubulointerstitial area, we did not evaluate the effect of FR167653 on the synthesis of proinflammatory cytokines in diabetic glomeruli.

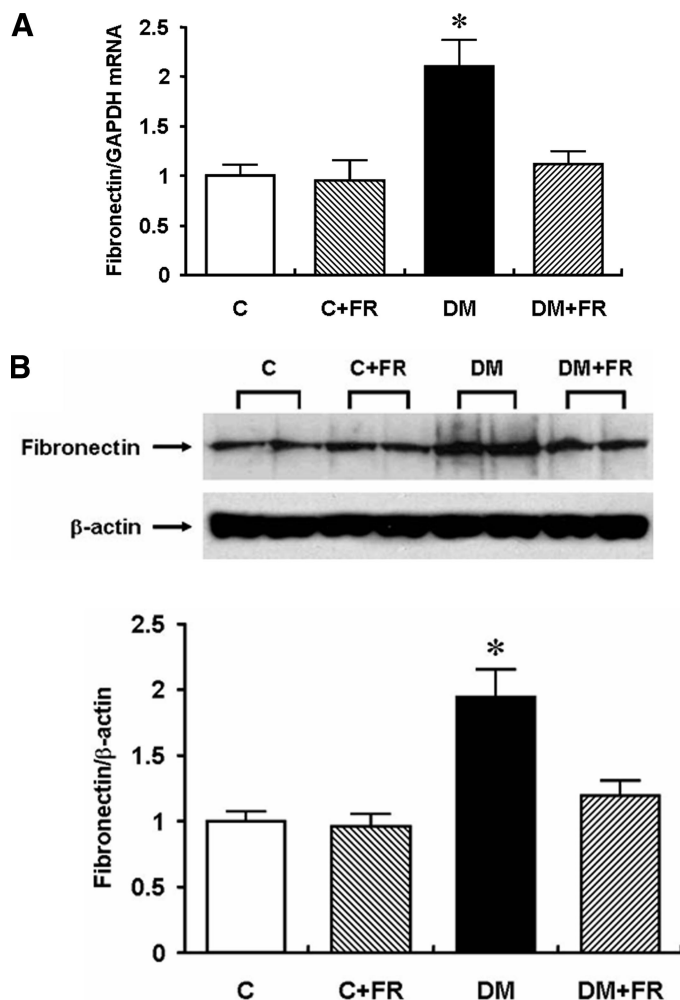


Fig. 4. *A*: fibronectin mRNA expression assessed by real-time PCR in C, C+FR, DM, and DM+FR glomeruli. The glomerular fibronectin-to-GAPDH mRNA ratio was 2.1-fold higher in DM compared with C rats, and this increment was inhibited by 89% with FR167653 treatment. On the other hand, FR167653 treatment had no effect on fibronectin mRNA expression in C rats. *B*: representative Western blot of glomerular fibronectin in C, C+FR, DM, and DM+FR groups (representative of four blots). Fibronectin protein expression was significantly increased in DM compared with C and C+FR glomeruli, and FR167653 treatment ameliorated this increment in glomerular fibronectin protein expression in the DM group. * $P < 0.05$ vs. other groups.

Besides the anti-inflammatory effects, recent studies have revealed that FR167653 is a selective p38 MAPK inhibitor and competes with ATP at the ATP-binding site of p38 MAPK (39). In addition, a previous study demonstrated that FR167653 did not affect the activities of other protein kinases, such as ERK1/2, JNK, PKC, or PKA (39). In this study, we also observed that the increases in ERK1/2 and JNK activities and PKC protein expression in HG-stimulated mesangial cells were not affected by FR167653 treatment, suggesting that the inhibitor effect of FR167653 was specific on the p38 MAPK pathway in cultured mesangial cells under diabetic conditions. In contrast to SB203580, another p38 MAPK inhibitor, it is known that FR167653 has no effect on cyclooxygenase-1 or -2 activity (39). By inhibiting the p38 MAPK pathway, renal NAD(P)H oxidase expression and superoxide formation were suppressed, and renal damage was ameliorated by FR167653 treatment in

Dahl salt-sensitive rats with heart failure, suggesting that the renoprotective effect of FR167653 was associated with the inhibition of oxidative stress (40). In addition, the study by Koshikawa et al. (21) demonstrated that p38 MAPK activation played an important role in podocyte injury in proteinuric glomerulopathies, including rat puromycin aminonucleoside nephropathy and mouse adriamycin nephropathy. Taken together, it seems that FR167653 can exert renoprotective effects by inhibiting the p38 MAPK pathway per se in various kidney diseases, even in which inflammatory cell infiltration is minimal or insignificant.

Apoptosis, usually known as programmed cell death, removes damaged or unwanted cells and has been impli-

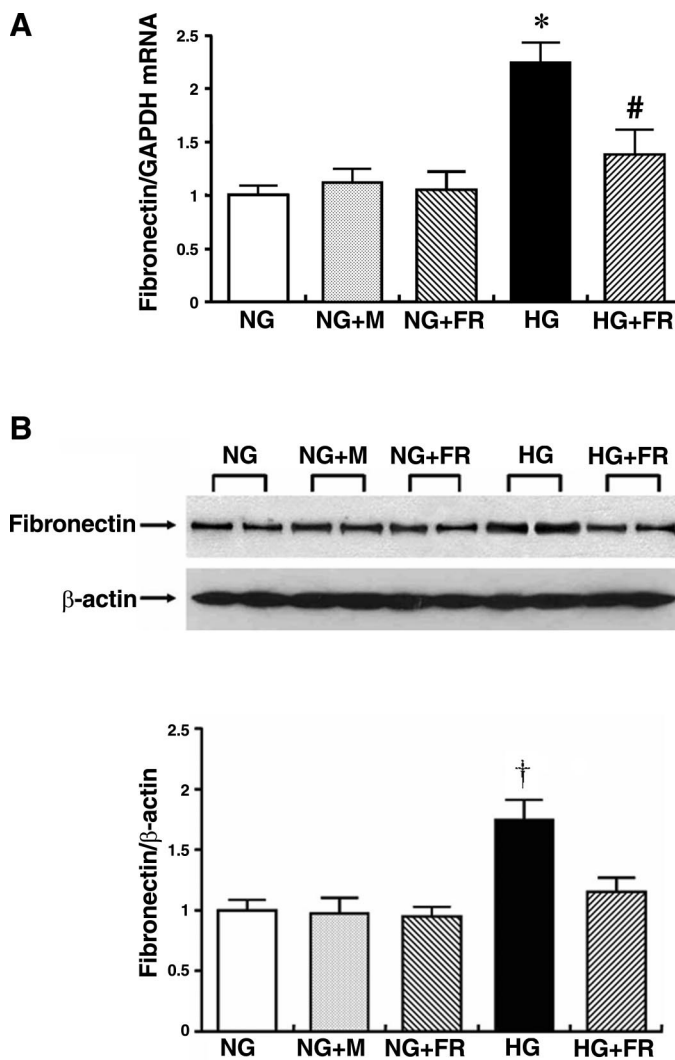


Fig. 5. *A*: fibronectin mRNA expression assessed by real-time PCR in cultured mesangial cells ($N = 5$). HG significantly increased fibronectin-to-GAPDH mRNA ratio in cultured mesangial cells, and this increment in fibronectin mRNA expression was inhibited by FR167653 treatment. In contrast, FR167653 and M had no effects on fibronectin mRNA expression in cultured mesangial cells. * $P < 0.01$ vs. NG, NG+M, and NG+FR groups; # $P < 0.05$ vs. HG group. *B*: representative Western blot of cellular fibronectin in cultured mesangial cells (representative of four blots). Fibronectin protein expression was significantly increased in HG-stimulated mesangial cells compared with NG cells, and FR167653 treatment ameliorated this increment in fibronectin protein expression in HG cells. † $P < 0.05$ vs. other groups.

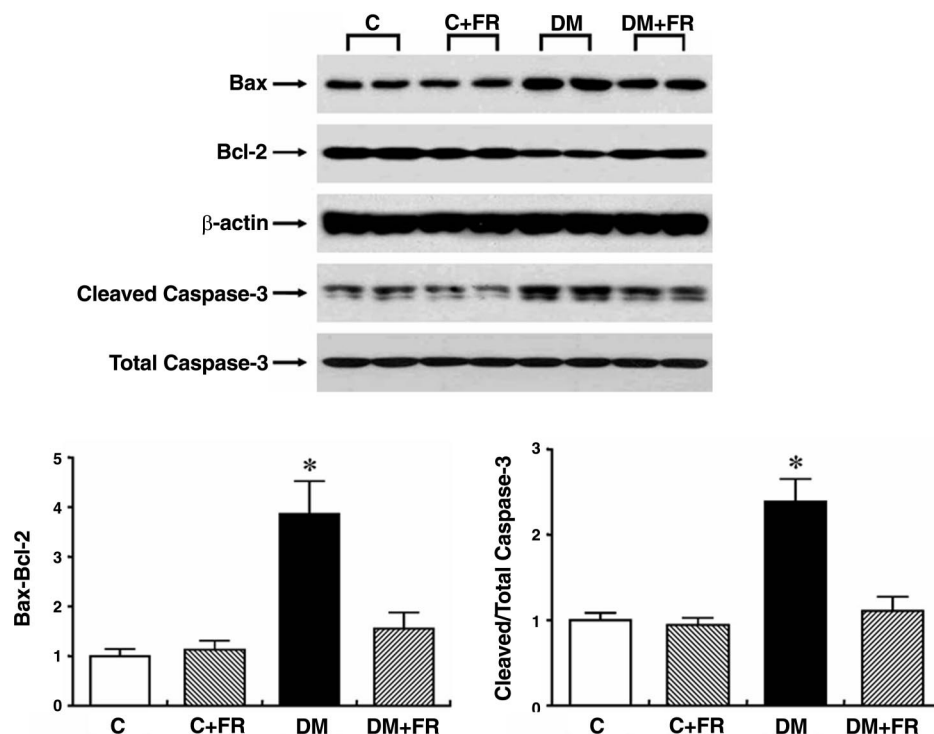


Fig. 6. Bax, Bcl-2, and cleaved caspase-3 protein expression assessed by Western blot in C, C+FR, DM, and DM+FR glomeruli (representative of four blots). Not only Bax protein, but also cleaved caspase-3 protein expression was significantly increased in DM glomeruli, and these increments were inhibited by FR167653 treatment. In contrast, Bcl-2 protein expression was significantly decreased in DM glomeruli, and FR167653 treatment ameliorated this decrement in Bcl-2 protein expression. * $P < 0.01$ vs. other groups.

cated in the pathogenesis of numerous diseases, such as malignancy, lupus erythematosus, and Alzheimer's disease (35). In addition, it has been documented in the course of various renal diseases, including diabetic nephropathy (6, 19, 38). Cell death by apoptosis is surmised to be involved in the process of mesangial cell loss in the late stage of diabetic nephropathy, suggesting that apoptosis may be a homeostatic mechanism regulating the glomerular cell pop-

ulation. Previous reports have demonstrated that high-glucose stimulated caspase-3 cleavage and DNA fragmentation in cultured mesangial cells (17, 27). Apoptosis of mesangial cells was also induced by hyperglycemia-related factors, methylglyoxal (25), advanced glycation end products (45), and transforming growth factor- β (33). On the other hand, numerous studies have investigated the signal transduction pathways mediating apoptosis in mesangial cells, and, as a

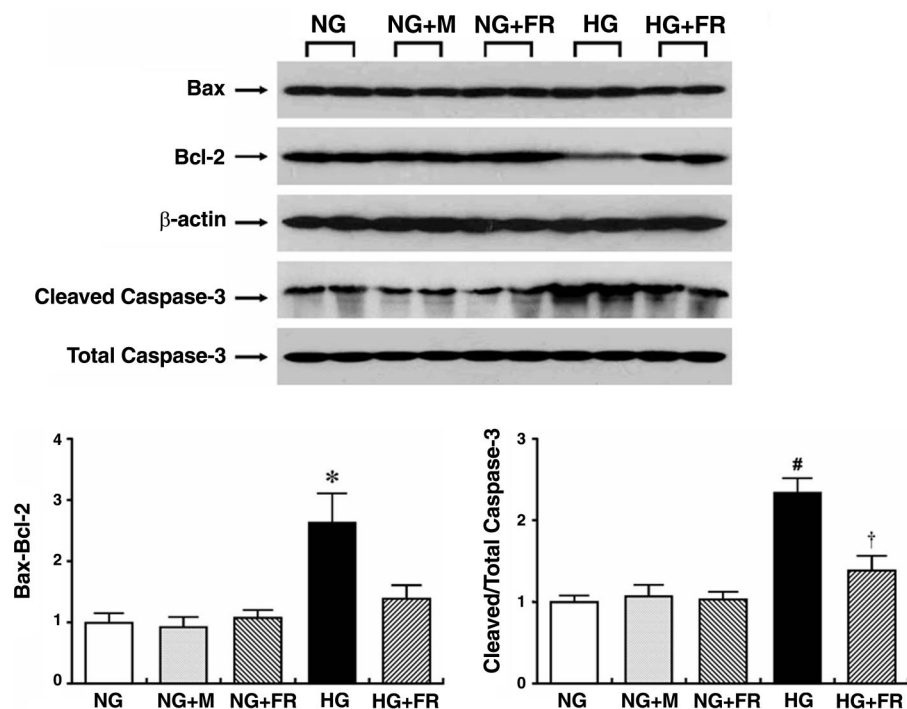


Fig. 7. Bax, Bcl-2, and cleaved caspase-3 protein expression assessed by Western blot in cultured mesangial cells (representative of four blots). Bcl-2 protein expression was significantly decreased, while cleaved caspase-3 protein expression was significantly increased in HG-stimulated mesangial cells, and these changes were inhibited by FR167653 treatment. On the other hand, Bax protein expression was increased in HG-stimulated mesangial cells, but did not reach statistical significance. * $P < 0.01$ vs. other groups; # $P < 0.01$ vs. NG, NG+M, and NG+FR groups; † $P < 0.05$ vs. HG group.

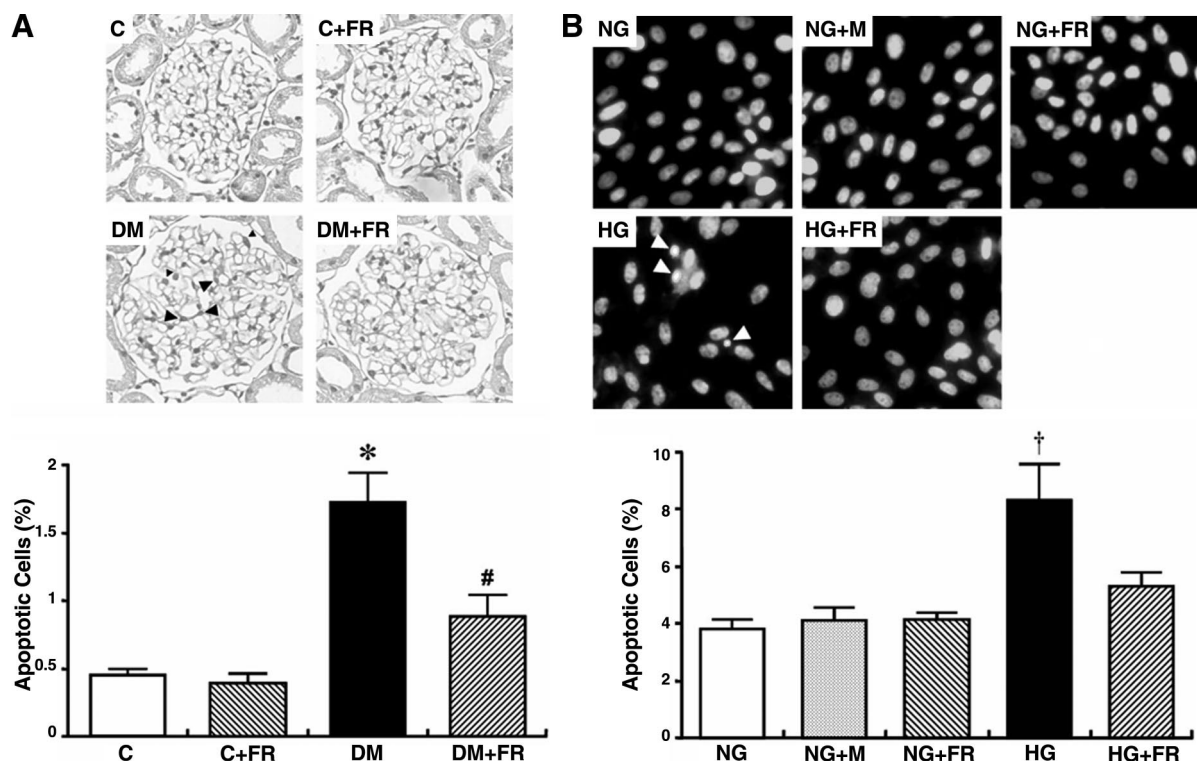


Fig. 8. Apoptosis assessed by terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling assay (A) and Hoechst 33342 staining (B). There was a significant increase in apoptotic cells (arrow heads) in DM glomeruli (large arrow heads, mesangial cells; small arrow heads, podocytes) and HG-stimulated mesangial cells compared with C glomeruli and NG cells, respectively, and this increase in apoptotic cells was ameliorated by FR167653, both in vivo and in vitro ($\times 400$). * $P < 0.01$ vs. C and C+FR groups; # $P < 0.05$ vs. DM group; † $P < 0.05$ vs. other groups.

result, the p38 MAPK pathway has been revealed to mediate mesangial cell apoptosis induced by several factors, including methylglyoxal (25), adrenomedullin (32), and homocysteine (37). However, the direct relationship between apop-

toxis and the p38 MAPK pathway has never been documented in diabetic glomeruli and in mesangial cells exposed to high glucose. Based on the results that treatment with a p38 MAPK inhibitor ameliorated apoptosis in diabetic glomeruli and

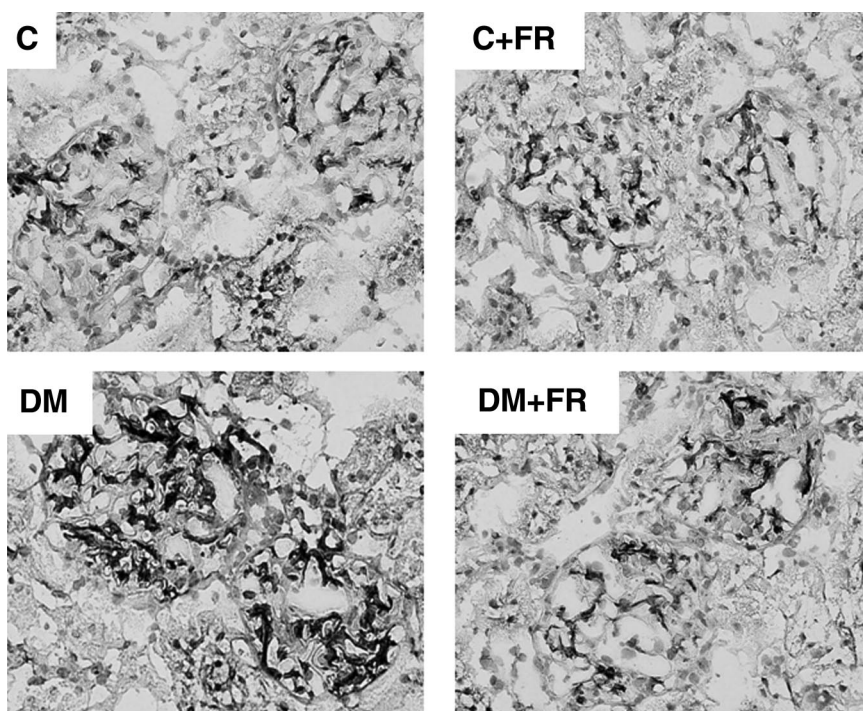


Fig. 9. Immunohistochemical staining for fibronectin in C, C+FR, DM, and DM+FR groups. There was a significant increase in glomerular fibronectin protein expression in DM compared with C and C+FR rats, and this increase was ameliorated by FR167653 treatment ($\times 400$).

high-glucose stimulated mesangial cells, the present study provides strong evidence that activation of p38 MAPK is closely linked to mesangial cell apoptosis under diabetic conditions.

In summary, FR167653 inhibited p38 MAPK activation and ameliorated fibronectin expression and apoptosis in diabetic glomeruli and in mesangial cells cultured under high-glucose conditions. These findings suggest that p38 MAPK could be a potential target for preventing nephropathy in diabetes.

GRANTS

This work was supported in part by the BK21 (Brain Korea 21) Project for Medical Sciences, Yonsei University, and the Korea Science and Engineering Foundation grant funded by the Korea government (Ministry of Science and Technology) (R01-2007-000-20263-0 and R13-2002-054-04001-0).

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